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Hypothesis

Formation of alternative proteasomes: Same lady, different cap?

Elah Pick *, Tali S. Berman

Department of Biology and Environment, University of Haifa at Oranim, Tivon 36006, Israel

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ABSTRACT

The 26S proteasome is thought to be a homogenous complex, consisting of a 20S proteolytic core and a 19S regulatory particle that is required for its activation.

Two groups have recently reported the activation of archeal 20S by a p97-related double-ring AAA+ ATPase complex, in a similar fashion to that reported for 19S. Since p97 is found in eukaryotes, the existence of a parallel setting in higher organisms is intriguing. Herein, we present supporting data and hypothesize that in eukaryotes, p97 and CSN form a promiscuous, hence hard-to-detect, "alternative cap", enabling the prompt and precise elimination of particular substrates.

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1. Introduction

Rapid response of cells to environmental changes or stress is key for all domains of life. Adjustment to new circumstances requires alterations of the cellular environment through degradation of unneeded proteins by the 26S proteasome, a master regulator of cellular quality control [1]. The 26S proteasome is a mega complex, arranged in 2 major assemblages: the 20S core particle (CP), a barrel-shaped complex, which is composed of 4 stacked heptameric rings (α,β,β,α), forming a tightly sealed gated cylinder, and the 19S regulatory particle (RP), which caps the 20S CP and controls/activates the entrance into the proteolytic cavity. The 19S RP (also referred to as "cap") is divided into lid and base assemblies. The lid is composed of 9 subunits (Rpn3, 5–9, 11, 12, and 15) and has a deubiquitinase enzymatic activity essential for proteolysis (donated by Rpn11). The base includes 2 large solenoid-shaped subunits (Rpn1, 2), 2 ubiquitin receptors (Rpn10, 13), and a motor AAA+ ATPase ring, which comprises 6 distinct subunits (Rpt1–6), and is required for the unfolding of substrates, opening the gate, and translocating substrates into the 20S CP [2]. Recent cryo-electron microscopy studies have provided evidence that several lid subunits interact directly with the AAA+ motor domain within several Rpt subunits (Rpn7 with Rpt2/6; rpn5/6 with Rpt3) [3–7] and even with the 20S (i.e. Rpn6) [8], suggesting that the Rpt1–6 ATPases ring is more static than was thought and might be motivated through substantial conformational rearrangements achieved by incorporation of the lid into the 26S holocomplex [5]. Activation of the 20S by the 19S involves interactions with a binding pocket within the α ring of the 20S CP, causing extensive conformational changes in the 20S and resulting in gate opening [9].

The ubiquitin proteasome system (UPS) plays an essential role in a variety of fundamental cellular processes. Not surprisingly, multiple proteasome activators (PA) exist in addition to the well-studied default 19S RP configuration. Three proteasome regulators, PA28/11S, PA200/Blm10, and ECM29, were found to compete with the 19S RP on the α-ring activation-binding site, in a non-ATP-dependent manner [10]. The PA28/11S consists of heteroheptameric (α,β,γ) rings, is induced by interferon-γ, associated with the 20S, and involved in immune response [11]. PA200/Blm10 activates the 20S and has a role in spermatogenesis, DNA repair, and other pathways [12]. ECM29, functions as a chaperon and stabilizer of proteasome 20S–19S interactions, as well as a negative regulator, which suppresses gate opening and causes proteasome blockage [13–16]. However, because interaction with the 20S CP (upon a specific cellular function or stress) could be transient, the list of proteasome regulators in eukaryotes might be longer and difficult

Abbreviations: Hb-Y-X, hydrophobic-tyrosine-X motif; PA, proteasome activator; CSN, COP9 signalosome; CRL, Cullin RING E3 ligase; PAN, proteasome-activating nucleotidase; Ribophagy, Ribosome autophagy; UbD, ubiquitin-binding domain; DUb, deubiquitinationg enzyme; UPS, ubiquitin proteasome system

* Corresponding author. Address: Department of Biology and Environment, Faculty of Natural Sciences, University of Haifa, Oranim, 36006 Tivon, Israel. Fax: +972 4 9539608.

E-mail address: elahpic@research.haifa.ac.il (E. Pick).

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to predict. One thing is clear, the proteasome is not assembled as one homogenous entity, and the exact number of PA-complexes and their respective roles is thus far, shrouded in mystery (Fig. 1).

Whereas both 19S substructures (base and lid) are found only in eukaryotes, a base-like subcomplex composed of AAA+ ATPases is found in several prokaryotes, with a conserved evolutionary role in acceleration of selective proteolysis. A few examples are ARC (*Rhodococcus erythropolis*), or MPA (*Mycobacterium tuberculosis*) that activate bacterial orthologs of the 20S proteasome [10,17]. Other bacterial examples are ClpA, C, or X, all of which activate the ClpP protease [18]. In archea, a homohexameric ring that is located proximally to the catalytic 20S subcomplex is known as PAN (proteasome-activating nucleotidase) [19]. PAN exhibits the highest homology with the eukaryotic Rpt-ring of ATPases, and shares more than 40% of amino acid similarity with them [20]. Interestingly, PAN is absent in several archea and is not required for viability of other archea in which the 20S exists and is required for protein degradation.

2. New insights regarding proteasome activators based on studies in archea

Recent studies have suggested a regulatory network of proteasome AAA+ ATPases across the archea kingdom. Bioinformatics analysis suggests that PAN is not alone, and that the putative number of proteasome ATPases in archea varies between 1 and 5, including the double ring AAA+ ATPase Cdc48/p97/VAT (p97), which is conserved from archea to humans. Interactions between archeal p97 and 20S CP, and formation of active proteasomes were found so far in 2 organisms, *Thermoplasma acidophilum* and *Methanosarcina Mazei* [21–23]. These newly described assemblages are actively involved in translocation of substrates into the proteolytic cavity [21,22]. Using bioinformatics tools, Barthelme and Sauer [16] showed that p97 is the only 20S CP potential partner in ~15% of archea, and that both p97 and PAN exist in similar rates in the remaining ~85% of analyzed genomes [16]. Interactions between the 20S and p97 involve a similar, conserved C-terminal hydrophobic-tyrosine-X motif (Hb-Y-X), which is also found in PAN and the Rpt1–6 ring [24,25]. In all of these interactions, the core complex is activated through docking of the activator into the α-ring-binding pockets within the 20S (Fig. 2) [21,24–26]. Interestingly, the Hb-Y-X motif exists not only in other archeal but also in eukaryotic p97 enzymes, suggesting a common mechanism for activation of proteolysis and that p97 has the potential of forming active proteasomes [16]. These new data raise questions regarding the evolutionary conservation of this unconventional architectural design (Fig. 2).

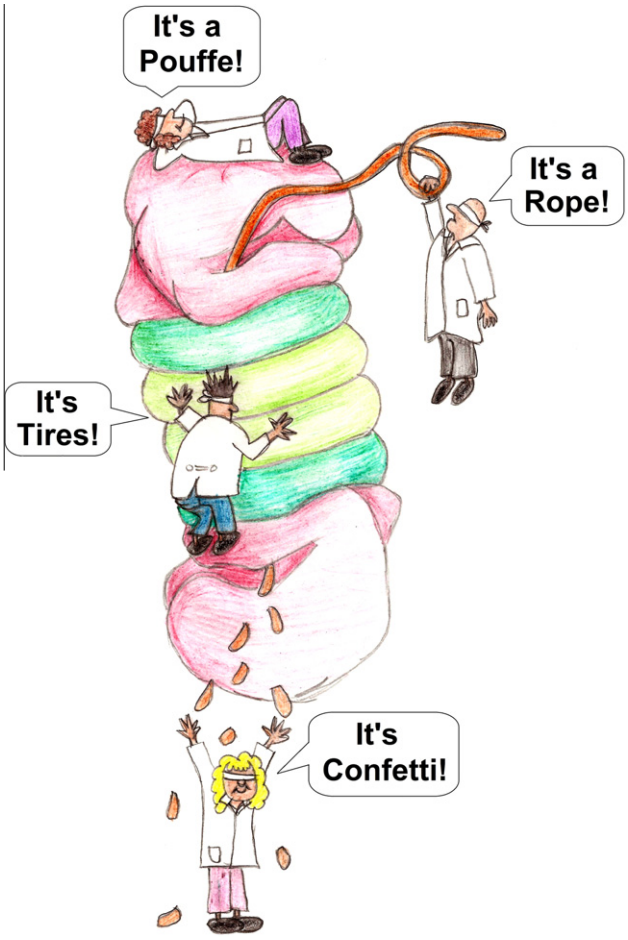

Fig. 1. Understanding proteasome function and regulation. Similar to the story about the blindfolded scientists who described an elephant differently according to the organ they held, the role and control systems of the proteasome are not fully understood in the scientific world.

3. p97 as a Proteasome activator: supporting information

Could it be that the eukaryotic p97 replaces the 19S and directly promotes selective protein degradation? In eukaryotes, p97 ATPase functions in a plethora of pathways with an important role in preparing proteins for degradation through the 2 key degradation apparatuses: the proteasome and the lysosome [27]. It participates in a wide range of cellular processes including cell-cycle regulation, response to DNA damage, ER degradation, and autophagy [28]. Together with its counterparts, p97 binds to polyubiquitinated substrates and uses ATP to unwind them. If required, p97 also functions to extract clients out of membranes or chromatin, and to eventually facilitate their degradation [29].

The eukaryotic p97 interacts with substrates that are covalently attached to a poly-ubiquitin chain through an array of p97-binding factors, such as the Ufd1/Npl4 counterparts that are required for extraction of substrates from chromatin and membranes, or members of the UbX family that harbor a ubiquitin-binding domain (UbD) and connect between p97 and substrates. Additionally, p97 also binds to an array of deubiquitinating enzymes (DUbs) such as the ovarian tumor protein Otu1, atx3 that regulates the degradation of misfolded ER proteins, Yod that is included in ER disclosure, or the Ubp3/Bre5 factors in budding yeast that are involved in ribosome autophagy (ribophagy) [30–33]. Interactions between 2 enzymes such as a DUb and an unfoldase (p97), could be explained by the need for recycling of client proteins. Yet, in addition to interactions with DUbs, the mammalian p97 interacts also with the proteasome [34], and may have the potential to play a role of PA, and to enable a prompt degradation of ubiquitinated proteins in situ. p97 and the 19S-RP have common characteristics: both include a molecular ATPase engine, interact with ubiquitin shuttles and with deubiquitinating enzymes, and both are involved in the regulation of proteolysis. Proteasome activation by p97 might explain the involvement of this molecule in the precise and rapid degradation of a wide array of proteasome substrates. Nevertheless, suggesting that eukaryotic p97 serves as an "alternative base" that activates the 20S-CP is unimaginable, because interactions between p97 and the eukaryotic proteasome have so far been confirmed only through the 19S and not directly with the 20S [29,34].

Finley and Matouschek have suggested 2 models that may explain the contribution of p97 to the eukaryotic proteasome [23]. The first model suggests that p97 was initially an integral part of

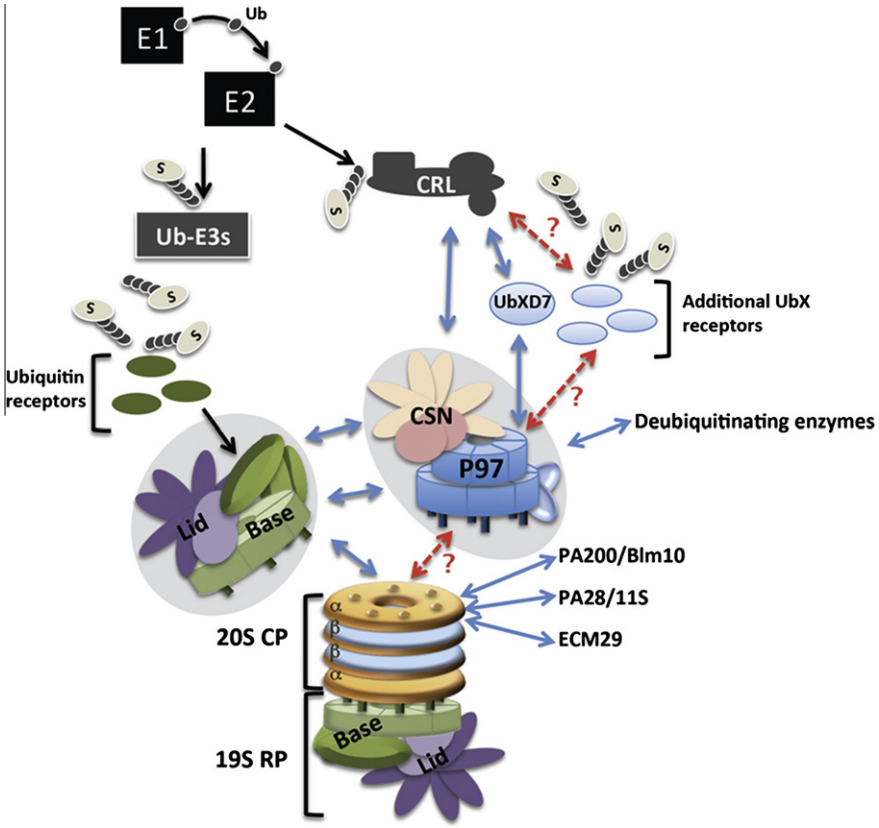

Fig. 2. A model for the formation of transient alternative proteasome activators required for the acceleration of ubiquitination/degradation of CRLs substrates. The 19S is a canonical proteasome activator. Ubiquitinated substrates are being shuttled by a ubiquitin receptor to the 19S proteasome for deubiquitination (Lid) and unfolding (Base), followed by activation of the 20S CP and proteolysis. The 19S is not always attached to the 20S, and 3 additional factors (PA28/11S, PA200/BLM10, and ECM29) regulate the 20S-CP conditionally, through interactions with similar residues, as does the 19S. The above mentioned model suggests an "alternative cap" assemblage, consisting of the CSN and p97 complexes, that regulate not only the ubiquitination of particular substrates, but also ubiquitin hydrolysis (Lid-like activity), and unfolding (Base-like activity). As a next step, alternative cap may cause to the activation of the 20S CP through interactions between the Hb-Y-X domain of p97 and specific residues within the alpha ring. Alternative PA may gather only transiently, on one end of the proteasome, and may be dependent on a specific substrate, a CRL, or a shuttling factor (such as UbXD7). Blue arrows show interactions confirmed in the literature; Red dashed arrows show predicted interactions.

the degradation machinery, however, the eukaryotic 19S took over the 20S-binding site, and interactions between p97 and the 20S were lost, while the rest of the ubiquitin–proteasome related functions of p97 were retained. The second model suggests that in eukaryotes, p97 still plays a role as a base-like complex, but for a subset of proteasome substrates that have not been identified thus far. In our hypothesis, we favor an integrated model, by which p97 has lost conventional interactions with the 20S, yet, it preserves promiscuous transient relationship with the 20S proteasome. The interactions with the 20S proteasome require additional counterparts that together enable to conditionally mimic the 26S.

4. p97 couples ubiquitination and degradation of a precise set of substrates

In archea, the variety of the proteasome components (including redundancy of ATPases) is critical for survival under stresses [22]. However, in eukaryotes, the highest level of diversity in the ubiquitin–proteasome pathway takes place mainly at the levels of ubiquitin E3 ligases, which confer specificity to substrates through a highly regulated mechanism [35,36]. Recently, several studies have shown that p97 is involved in the regulation of ubiquitination through direct interactions with ubiquitin E3 ligases, including several members of the cullin-RING E3 ligase (CRLs) family [37–42]. CRLs are the largest class of E3 ligases. Seven different cullin scaffold proteins exist in humans, each of them serve as a building block for the assembly of tens or more multi-subunit CRLs. Cullins interact with a RING domain protein Rbx1/Rbx2, via its C-terminus and to a cullin-specific substrate adaptor protein via its N-terminus (with one exception of Cul3) [35,36]. Adaptors bind substrate-receptors, which in turn recruit substrates for ubiquitination. CRLs activity is highly regulated, and complexes are active when covalently attached to the ubiquitin-related protein, Nedd8. Neddylation/deneddylation cycles are required for the proper regulation of CRL function. A key regulator of CRLs activity is the COP9 signalosome (CSN), an 8-subunit complex that shows 1:1 paralogy with each of the proteasome lid subunits [43]. Paralogy between the CSN and the lid is also reflected by the enzymatic function: while Rpn11 deconjugates ubiquitin from proteasome substrates, its paralog in the CSN, Csn5 hydrolyses Nedd8/Rub1, the closest paralog of ubiquitin from cullins [44]. The knowledge about regulation of CRLs by the CSN has recently been expanded, when a few independent groups found that the CSN controls CRLs activity also in a non-enzymatic fashion [45–47].

Similar to the CSN, p97 also regulates CRLs activity, through its counterpart UbXD7, a member of the UbX family. UbXD7 interacts with the neddylated form of CRL2 and CRL4 and mediates their interactions with p97 through its conserved ubiquitin-interacting motif (UIM). It also interacts with polyubiquitin chains through its UbX domain, and may act as a ubiquitin receptor through this domain, and inhibit deneddylation through direct interactions with Nedd8 (Fig. 2) [37–39].

Degradation of several CRL substrates is mediated by p97: the hypoxia-inducible factor Hif-α, substrate of CRL2Elo B-C/VHL, the mitotic kinase Aurora B, and the yeast RNAPII catalytic subunit (Rpb1), which are ubiquitinated by CRL3Klhl21 and CRL3Elc1/Ela1, respectively, as well as the replication licensing factor Cdt1 which is targeted by CRL4ADdb1/Cdt2 [37–39,42]. However, degradation of Cdt1 does not require interactions with UbxD7, and might involve a yet undefined UbX receptor ([48], Fig. 2).

5. Performing alternative caps to the proteasome: old model with new insights

Similar to p97 and the "alternative-base" model [23], a model for the CSN complex as an "alternative lid," which modulates the activity and efficiency of substrate ubiquitination and degradation in a single conglomerate, has been previously suggested and remains to be elucidated [49]. The 2 complexes, CSN and p97 share few characteristics: both regulate the UPS, both interact with the proteasome directly [20,34,50,51], both regulate CRLs (CSN inhibits CRLs activity in vitro, and is, unexpectedly a positive regulator in vivo [51]; p97 regulates CRLs through UbXD7 and may also have positive and negative regulatory roles depending on substrates or the performed study [36]), and both CSN and p97 are involved in pleiotropic biological functions.

A previous study has shown that mammalian p97 interacts with the CSN complex in an ATP-dependent manner, and may form an ATP-dependent complex that resembles the 19S regulatory particle [52]. Both deubiquitinase and deneddylase activities of the CSN are required for the binding between p97 and polyubiquitinated substrates [52]. In addition, studies conducted in human B8 fibroblasts cells have shown that CSN interacts directly with the proteasome and competes with the lid, thereby impacting the peptidase activity of the proteasome in vitro [48]. Considering all of the above-mentioned data, it is intriguing to predict that p97 activates proteasomes through transient interactions between its HY-b-X motif and the 20S on one side [21,22], and on the other side accomplishes roles such as unfoldase and deubiquitinase through interactions with the CSN (Fig. 2) [52]. It will be interesting to go one step further and revive the old "alternative lid" model; however, the new model requires substitution of the base, and might imprison a CRL complex, together with shuttling molecules such as a UbX protein in-between (Fig. 2), while the "other side" of the proteasome may still bind conventional 19S-RP. Such a conditional assemblage would be very hard to detect using conventional structural methodologies. Obviously, this predicted proteasome subcomplex is not commonly found. Perhaps, it performs promiscuous interactions with the 20S, and requires regulation of a specific substrate, ligase, or shuttling factor. Nevertheless, such a mechanism may enable the precise and prompt elimination of substrates.

6. Concluding remarks

The 2 protein complexes, CSN and p97 possess structural and functional paralogy with the 19S RP lid and base subcomplexes, respectively. p97 and the Rpt1–6 ring of ATPases function as unfoldase, while the CSN and the lid function in hydrolysis of members of the ubiquitin family. Just as in the aforementioned models, we also suggest that the eukaryotic p97 (as well as the CSN complex) have evolved a new function and lost the ability to function as a proteasome regulatory particle. Nevertheless, we hypothesize that p97 is still able to play a promiscuous role, and moonlight within the proteasome under certain conditions, a phenomenon that might represent an intermediate phase for the evolvement of this complex. Such as other moonlighting proteins, this interaction is transient, and is difficult to detect. Moonlighting of paralog complexes in the proteasome, playing as "conventional homologs" could elucidate a sophisticated mechanism to elevate the complexity within the ubiquitin proteasome system and provide an additional level of specificity by building distinct types of proteasomes from existing regulators, without requiring extension of the genome.

We assume that proteasome activation by a complex such as the p97/CSN could be unstable under standard laboratory conditions. Future analysis should therefore be performed in response to diverse stimuli, or environments (such as stressors), followed by analysis of conditional protein–protein interactions that may bring ubiquitination in close proximity with the proteasome, modulating efficient turnover of selected CRL complexes and their substrates.

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